PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Kenji MIYAMOTO, et al.

Docket No: Q95907

Appln. No.: 10/585,417

Group Art Unit: 1623

Confirmation No.: 4711

Examiner: Scarlett Y. GOON

Filed: April 10, 2007

For: HYALURONIC ACID DERIVATIVE AND DRUG CONTAINING THE SAME

DECLARATION UNDER 37 C.F.R. § 1.132

MAIL STOP AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

(1) I, Kenji Miyamoto, hereby declare and state:

THAT I am a citizen of Japan;

THAT I received a Master's degree from the Graduate School of Science, Science University of Tokyo in March 1991, and I did research in the field of peptide chemistry;

I have been employed by Seikagaku Corporation since 1992, where I engaged in research related to chemical modification of glycosaminoglycan in Seikagaku Corporation's Central Research Laboratories.

(2) I, Yousuke Yasuda, hereby declare and state:

THAT I am a citizen of Japan;

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THAT I received a Master's degree in engineering science from the Graduate School of Osaka University in March, 1995, and I did research in the field of protein engineering;

I have been employed by Seikagaku Corporation since 1995, where I engaged in research mainly on chemical synthesis in Seikagaku Corporation's Central Research Laboratories.

(3) I, Keiji Yoshioka, hereby declare and state:

THAT I am a citizen of Japan;

THAT I received a Master's degree in pharmaceutical sciences from the Graduate School of University of Shizuoka in March, 1998, and I did research in the field of peptide chemistry; I took a license of pharmacist in 1996.

I have been employed by Seikagaku Corporation since 1998, where I engaged in pharmacological testing of chemical compounds and performance studies of medical devices. Since 2001, I have been engaged in the development of therapeutic agents for osteoarthritis of the knee in Seikagaku Corporation's Central Research Laboratories.

THAT we personally conducted or supervised the conduct of the following experimentation to show that the hyaluronic acid compound of U.S. Application Serial No. 10/585,417 (herein after "the substance of the present invention") exhibited unexpectedly superior analgesic effect as compared to the test substances.

THAT we carried out the following experimentation from July 2009 through December 2009 in Seikagaku Corporation's Central Research Laboratories in order to further confirm the unexpectedly superior properties of the claimed invention of U.S. Application Serial No.10/585,417 and to demonstrate the repeatability of the claimed invention.

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Experimental Procedure:

I. Synthesis procedure

Three synthesis Examples were prepared and tested as explained below.

The degree of substitution of diclofenac moiety was calculated by the following method (1) or (2).

(1) Method by ultraviolet spectrophotometry

Absorbance of the substance (aminopropanol-diclofenac hydrochloride) prepared according to Example 18 of U.S. Application Serial No.10/585,417 in various concentrations were measured at 280nm to make a calibration curve. The absorbance of aminoethanol-diclofenac introduced sodium hyaluronate (the substance(1-2)) was measured at 280nm and the degree of substitution of diclofenac moiety was calculated based on the calibration curve.

To determine the degree of substitution in diaminoethane-diclofenac introduced sodium hyaluronate (the substance(2-2)), the same method described above was employed, by using the calibration curve of the substance (diaminopropane-diclofenac hydrochloride) (Example 40 of U.S.Application Serial No.10/585,417).

(2) Method by 1H-NMR

Ethyleneglycol-diclofenac-introduced sodium hyaluronate (3-2) obtained in Synthesis Example 3 was subjected to 1H-NMR. A degree of substitution of diclofenac moiety was calculated based on a ratio of the signal strength for 3H at 7.2 to 7.4ppm obtainable in diclofenac moiety and the signal strength assigned to N-acetyl group of N-acetyl glucosamine.

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1. **Synthesis Example 1**

Synthesis of aminoethanol-diclofenac-introduced sodium hyaluronate (the substance of the present invention)

(1-1) Boc-aminoethyl bromide was prepared in the same manner as in Reference Example 5 of U.S. Application Serial No. 10/585,417. Then, in accordance with the procedure in Example 38 of U.S. Application Serial No. 10/585,417, aminoethanol-diclofenac hydrochloride was prepared.

(1-2) In 116 mL water /145 ml 1,4-dioxane, 1 g (2.5 mmol/disaccharide unit) of sodium hyaluronate having a weight average molecular weight of 800,000 was dissolved, and then 1.5 mL of 1 mol/L aqueous hydroxysuccinimide (HOSu) solution, 1.5 mL of 0.5 mol/L aqueous 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (water-soluble carbodiimide hydrochloride, WSCI-HCl) solution and 7 mL of the solution (water : 1,4-dioxane = 1:1) of 281.8 mg (0.75 mmol) of aminoethanol-diclofenac hydrochloride obtained in the above 1-1 were added thereto in this order at ambient temperature, followed by stirring overnight.

To the reaction solution, 15 mL of 5% aqueous sodium hydrogen carbonate solution was added, followed by stirring for 3.5 hours. After neutralizing the reaction solution by adding 430 μL of 50% acetic acid, 5 g of sodium chloride was added thereto, followed by stirring. The mixture was precipitated by adding 1 L of ethanol, and the precipitate was collected by filtration and washed twice with 85% ethanol, twice with ethanol and twice with diethyl ether and dried at room temperature overnight under reduced pressure to give 1.079 g of aminoethanol-diclofenac

introduced sodium hyaluronate in the form of a white solid. The degree of substitution of diclofenac was 17.0 % as determined by means of ultraviolet spectrophotometry.

2. **Synthesis Example 2**

Synthesis of diaminoethane-diclofenac-introduced sodium hyaluronate (Diamide substance)

(2-1) In 5 mL of dimethylformamide (DMF), 1.46 g (4.93 mmol) of diclofenac and 2.67 g (7.05 mmol) of *O*-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate was dissolved, and then 7 mL of DMF solution of 755 mg (4.71 mmol) of N-Boc-1,2-diaminoethane, 1.72 mL (9.88 mmol) of N,N-diisopropylethylamine were added thereto, followed by stirring for 140minutes. After adding ethyl acetate to the reaction solution, the mixture obtained was separated. The organic layer was washed with a 5 % citric acid solution, a saturated solution of sodium hydrogen carbonate and saturated solution of sodium chloride in turn. After drying the organic layer over sodium sulfate, ethyl acetate was removed under reduced pressure. Thus obtained residue was purified by silica gel column chromatography (eluent; chloroform) to give 1.73 g of substance (2-1) (84 %).

(2-2) To 463 mg (1.06 mmol) of the above substance (2-1), 5 mL of 4 mol/L hydrochloric acid/ethyl acetate was added at ice-water temperature, followed by stirring for 2 hours. The obtained precipitate was washed with hexane and diethylether in turn and dried under reduced pressure to give 395 mg of substance (2-2) (100 %).

(2-3) In 22.5 mL water /22.5 mL 1,4-dioxane, 200 mg (0.5 mmol/disaccharide unit) of sodium hyaluronate having a weight average molecular weight of 800,000 was dissolved, and

then 0.2 mL of 2 mol/L aqueous hydroxysuccinimide (HOSu) solution, 0.2 mL of 1 mol/L aqueous WSCl-HCl solution and 0.6 mL of aqueous solution of 74.8 mg (0.2 mmol) of the above obtained substance (2-2) were added thereto in this order, followed by stirring overnight. To the reaction solution, 3 mL of 5% aqueous sodium hydrogen carbonate solution was added, followed by stirring for 4 hours. After neutralizing the reaction solution by adding 86 μL of 50% acetic acid, 3.4 mL of aqueous solution of 1 g of sodium chloride was added thereto, followed by stirring. The mixture was precipitated by adding 148 mL of ethanol, and the precipitate was washed twice with 90% ethanol, twice with ethanol and with diethyl ether in turn and dried at room temperature overnight under reduced pressure to give 190 mg of diaminoethane-diclofenac-introduced sodium hyaluronate in the form of a white solid. The degree of substitution of diclofenac was 15.9 % as determined by means of ultraviolet spectrophotometry.

3. Synthesis Example 3

Synthesis of ethyleneglycol-diclofenac-introduced sodium hyaluronate (Diester substance)

(3-1) In 3 mL dichloromethane/0.5 mL DMF, 502.8 mg (1.70 mmol) of diclofenac was dissolved, and then 2.5 mL of dichloromethane solution of 294.3 mg (1.71 mmol) of 2-iodoethanol, 40.7 mg (0.333mmol) of 4-(dimethylamino)pyridine and 393.5 mg (2.05 mmol) of WSCI-HCl were added thereto at ice-water temperature, followed by stirring for 50 minutes. After adding ethyl acetate to the reaction solution, the mixture was separated. The organic layer was washed with a 5 % citric acid solution, a 5 % aqueous sodium bicarbonate solution, distilled water and a saturated solution of sodium chloride in turn. After drying with

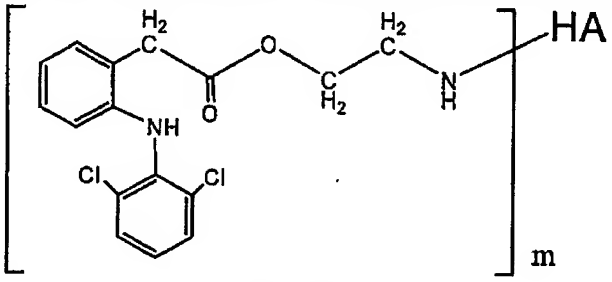
magnesium sulfate, ethyl acetate was removed under reduced pressure. The thus obtained residue was purified by silica gel column chromatography (hexane: ethyl acetate = 10:1) to give 586.5 mg of substance (3-1) (77 %).

(3-2) In accordance with the procedure disclosed in EXAMPLE D of U.S. Patent No. 4,851,521 (Preparation of the salt of tetrabutylammonium of hyaluronic acid), tetrabutylammonium hyaluronate was prepared using sodium hyaluronate having a weight average molecular weight of 800,000. To 4.62 g of dimethylsulfoxide (DMSO) solution of 62.1 mg (0.1mmol) of tetrabutylammonium hyaluronate, 2.70 g of DMSO solution of 27.0 mg (0.060 mmol) of the above obtained substance (3-1) was added, followed by stirring for 16 hours. To the reaction mixture, 0.8 mL of 2.5 mol/L sodium chloride solution was added and the mixture was precipitated by adding 45 mL of acetone. The precipitate was washed three times with 83 % aqueous acetone and three times with acetone and dried overnight under reduced pressure. The precipitate was dissolved with 10 mL of a 1% sodium chloride aqueous solution and then the solution was precipitated by adding 50 mL of acetone. The precipitate was washed twice with 83 % aqueous acetone and three times with acetone and dried overnight under reduced pressure to give 36.6 mg of ethyleneglycol-diclofenac-introduced sodium hyaluronate in the form of a white solid. The degree of substitution of diclofenac was 17.2 % as determined by ^{1}H-NMR.

The substances obtained in Synthesis Examples 1, 2 and 3 are a partially modified hyaluronic acid represented by each Formulael, 2 and 3, respectively. In Formulae 1, 2 and 3, HA represents a partial structure of the hyaluronic acid, which is represented by Formula 4 as a

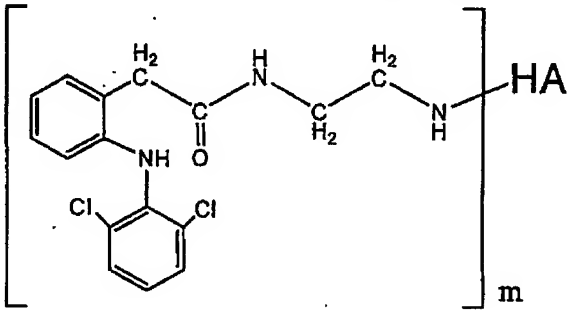
carboxy residue of disaccharide unit of hyaluronic acid, and m represents modification ratio of the carboxyl group to a total of carboxyl group of the hyaluronic acid.

The substance obtained in Synthesis Example 1 is represented by Formula 1, and m is 17.0%.



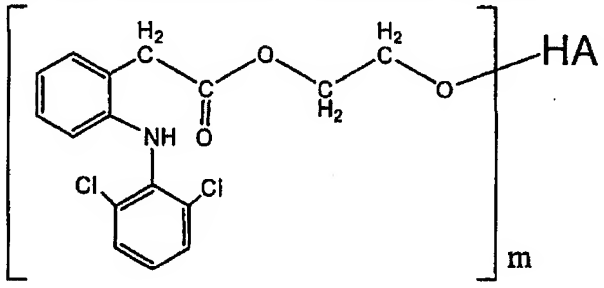
Formula 1

The substance obtained in Synthesis Example 2 is represented by Formula 2, and m is 15.9%.

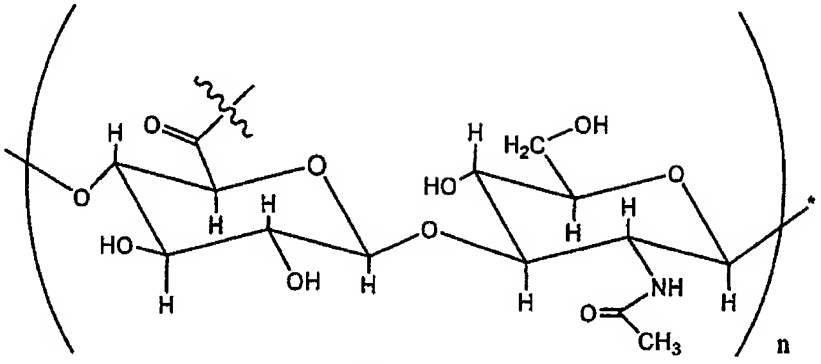


Formula 2

The substance obtained in Synthesis Example 3 is represented by Formula 3, and m is 17.2%.



Formula 3



Formula 4

II. Effect of the present invention

In accordance with the procedure of Examples 44 and 47 of U.S. Application Serial No. 10/585,417, the effects of intra-articular injection of the three substances obtained in the above synthesis examples on a 1% silver nitrate-induced pain model in rats were examined.

1. Testing substances:

(I) 1% PBS solution of the substance obtained in Synthesis Example 1 (the substance of the present invention)
(II) 1% PBS solution of the substance obtained in Synthesis Example 2 (diamide substance)
(III) 1% PBS solution of the substance obtained in Synthesis Example 3 (diester substance).
(IV) PBS (control)

2. Evaluation Method

In accordance with the procedure of Example 44 of the present application, pain score and weight loading rate measured with a weighting activity analgesia meter (manufactured by Tokken Inc.) were examined.

3. Pain Score:

0: Normal;
1: Mild claudication;
2: Severe claudication;
3: Walking on three legs

4. Calculation Formula of Weight loading rate:

Weight loading rate (%) = the mean value of weight loading on inflamed leg (g) / body weight (g) × 100

5. Administration Procedure:

Arthritis was induced by administering 1% of silver nitrate solution into the knee joint cavity of the left hind paw of rat (Crj:SD (SPF), male, 5-weeks-old) at a dose of 50 μL/joint. One day after induction, the rats were divided into 4 groups (9 rats for each group) on the basis of pain score and weight loading rate. Each of the above test substances was administered into the knee joint cavity of the left hind paw of the rats at a dose of 500 μg/50 μL/ joint. In addition, the dose of PBS was 50 μL/joint. Evaluation of the rats' walking was carried out on the first day, the second day and the third day after administration by using the pain score and the weight loading rate under blind conditions.

III. Results

A line graph of pain score and a line graph of weight loading rate are shown below.

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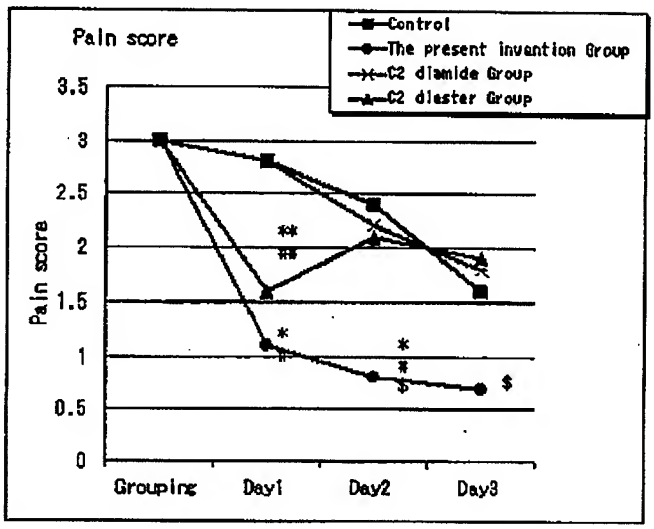

Mean±SE (N=9)

**p<0.01, *p<0.05: Steel-Dwass test (The present invention Group, C2 diester Group vs Control)

##p<0.01, #p<0.05: Steel-Dwass test (The present invention Group, C2 diester Group vs C2 diamide Group)

$p<0.05: Steel-Dwass test (The present invention Group vs C2 diester Group)

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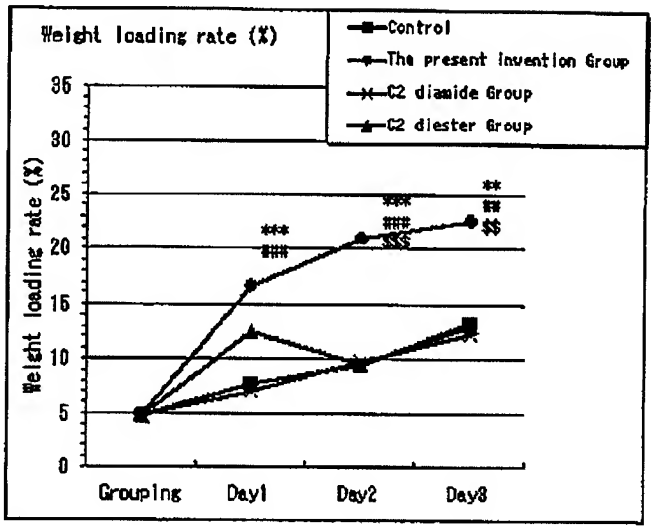

Mean± SE (N=9)

***p<0.001, **p<0.01: Parametric Tukey test (The present invention Group vs Control)
###p<0.001, ##p<0.01: Parametric Tukey test (The present invention Group vs C2 diamide Group)
$$$p<0.001, $$p<0.01: Parametric Tukey test (The present invention Group vs C2 diester Group)

In the above graphs:

- (Control) represents the group of rats treated with PBS
- (Synthesis Example 1 - The present invention) represents the group of rats treated with the compound of the present invention (the combination of an amide bond and an ester bond). Namely, hyaluronic acid is bound to aminoethanol via an amide bond, and aminoethanol is bound to diclofenac via an ester bond.
- (Synthesis Example 2 - C2 diamide Group) represents the group of rats treated with a diamide compound. Namely, hyaluronic acid is bound to diaminoethane via an amide bond, and diaminoethane is bound to diclofenac via an amide bond.
- (Synthesis Example 3 - C2 diester Group) represents the group of rats treated with a diester compound. Namely, hyaluronic acid is bound to ethyleneglycol as a spacer via an ester bond, and ethyleneglycol is bound to diclofenac via an ester bond.

As shown in the pain score graph, the substance of the present invention (Synthesis Example 1) exhibited a significant improvement effect on pain from Day 1 to 2, as compared to the control. On the other hand, C2 diester (Synthesis Example 3) treated animals showed significantly lower pain scores on Day 1 compared with the control, however, that analgesic effect was no longer seen from Day 2. In the C2 diamide (Synthesis Example 2) treated animals, the pain score at each time point of evaluation was comparable to that of the control.

As shown in the weight loading rate graph, the substance of the present invention (Synthesis Example 1) exhibited a significant improvement effect on weight loading rate from Day 1 to 3, as compared to the control, and this effect was observed at all time points until Day 3. On the other hand, in the C2 diamide (Synthesis Example 2) and C2 diester (Synthesis Example 3) groups, there were no significant increases in the weight loading rates at any time points from Day 1 to 3, as compared to the control.

Based on the above results, the substance of the present invention exhibited unexpected and significant analgesic effects due to a specific binding mode (amide linkage-ester linkage) as a technical feature of the present invention.

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I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 2010/8/24

Kenji Miyamoto
Kenji MIYAMOTO

Date: 2010/8/24

Yousuke Yasuda
Yousuke YASUDA

Date: 2010/8/24

Keiji Yoshioka
Keiji YOSHIOKA